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TITLE: The Role of Human Spectrin SH3 Domain Binding Protein 1
(HSSH3BP1) in Prostatic Adenocarcinoma

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Edward C. Jones, M.D.

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Incorporated
Albany, New York 12229

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13. ABSTRACT (Maximum 200 Words) Prostate cancer is one of the leading causes of cancer-related deaths in the United States and a leading diagnosed cancer in American men. Newly diagnosed cases of prostate cancer approach rapidly the number of 200,000 cases per year. Genetic alterations of tumor suppressor genes are one of the most common causes of prostate cancer tumorigenesis. Our group identified Hssh3bp1 as a candidate prostate tumor suppressor gene. In this research we are testing the tumor suppressor function of the candidate gene in prostatic adenocarcinoma using <i>in vitro</i> and <i>in vivo</i> assays. Using developed cell lines expressing Hssh3bp1 we established a candidate region of Hssh3bp1 responsible for growth regulation. Within this region we mapped a tyrosine phosphorylation site by c-Abl tyrosine kinase <i>in vitro</i> . This year we established that the same site is phosphorylated in transfected cells and that the phosphorylation of Hssh3bp1 is tightly regulated. Further work is under way to develop a detail understanding what is the mechanism of growth regulation by Hssh3bp1 and c-Abl kinase. It is hoped that this work will help us to understand prostate cancer tumorigenesis and ultimately lead to better diagnosis and therapy strategies.				
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Introduction

Prostate cancer is one of the leading causes of cancer-related deaths in the United States (over 41,000 per year) and a leading diagnosed cancer in American men (43% of all diagnosed cancer in men). Newly diagnosed cases of prostate cancer approach rapidly the number of 200,000 cases per year. Genetic alterations of tumor suppressor genes are one of the most common causes of neoplastic transformation leading to tumorigenesis including prostate cancer tumorigenesis. Inactivation of one or more tumor suppressor genes is thought to be the most common cause of prostatic adenocarcinoma. Our group identified such candidate tumor suppressor gene. The gene was originally named Hssh3bp1 for its binding properties to spectrin SH3 domain (*human spectrin SH3 domain binding protein 1*).

In this research we propose to test the tumor suppressor function of a candidate gene in prostatic adenocarcinoma using *in vitro* and *in vivo* assays. The work is directed at understanding what is the mechanism of loss of Hssh3bp1 expression in prostatic cells lines and tumors, and will test potential tumor suppressive role of Hssh3bp1 in nude mice. Hssh3bp1 is a potential regulator of macropinocytosis. Macropinocytosis can be upregulated by growth factors, which in turn promote tumor growth; we propose that Hssh3bp1 is a negative regulator of macropinocytosis and cell growth. To learn more about possible mechanisms of Hssh3bp1 tumor suppressor function we will determine whether Hssh3bp1 mutations affect macropinocytosis of prostate cells and determine molecular events underlying this effect. Although it is possible that Hssh3bp1 is not involved in biogenesis of prostate cancer, after completion of the proposed work we will know more about the function of the protein in human prostate. On the other hand, with the identification of Hssh3bp1 as a tumor suppressor gene in prostate cancer, it is likely to lead to subsequent hypotheses and research on the Hssh3bp1 role in prostate tumorigenesis. This, in turn, is likely to lead to a better diagnosis, treatment, and possibly prevention of this deadly disease.

Body

The Role of Human Spectrin SH3 domain Binding Protein 1 (Hssh3bp1) in Prostatic Adenocarcinoma

Introduction.

This year was the transition year for our laboratory. The laboratory was moved from our previous location at the NYS Institute for Basic Research, Staten Island, NY to the Linsley F. Kimball Research Institute (LFKRI). LFKRI is part of the New York Blood Center (NYBC), New York, NY. Upon this move we established the Laboratory of Cell Signaling at LFKRI. Our move to LFKRI included closing down the laboratory at NYS Institute for Basic Research, Staten Island, NY, moving the laboratory equipment and research samples to NYBC, setting the new laboratory at NYBC, and hiring new people. As a result of this move this grant was subcontracted from the Research Foundation of Mental Hygiene, Inc. to NYBC. In addition the grant was extended for one year with no additional cost. As a result of the transfer specific research program was established to be performed at the New York Blood Center, NY.

The research program to be performed at the New York Blood Center, NY is focused on completion of Tasks 1 and 3 of the grant. Task 2 has been completed.

Original Task 1. Determine whether hssh3bp1 is not expressed in some prostate tumors due to presence of mutations.

- a. Search for mutations of the hssh3bp1 cDNA and gene in prostate tumor cell lines and primary prostate tumors (30 cases).
- b. Determine pattern of hssh3bp1 expression in primary prostate tumors. Correlate the pattern of hssh3bp1 expression with the tumor grade and stage (100 cases).

Work to be performed:

Task 1a. We will sequence the Hssh3bp1cDNA isolated from the collected prostate biopsies specimens. The sequence data will be analyzed for mutations that may lead to loss of function of Hssh3bp1.

Task 1b. We will analyze expression of Hssh3bp1 by immunochemistry in prostate tissue array.

Original Task 3. To identify a potential mechanism and a signal transduction pathway involved in the tumor suppression function of Hssh3bp1.

- a. Determine the role of Hssh3bp1 mutations in macropinocytosis of prostate cell lines.
- b. Determine the role of growth factors, PI3-kinase, and the 200-kDa spectrin-like protein in the function of Hssh3bp1.

Work to be performed:

Task 3. As reported in our last year progress report c-Abl tyrosine kinase is a major regulator of Hssh3bp1 function so we decided to focus our efforts on the c-Abl kinase pathway. We will determine whether Hssh3bp1 is phosphorylated by c-Abl kinase, and whether, Hssh3bp1 regulates apoptosis of LnCAP cells.

Progress towards Aim 1 (Task 1).

We have delayed experiments involving research on Aim 1. This delay is due to two factors: a, due to delay in subcontracting the grant to NYBC and subsequent delay in hiring the laboratory personnel; b, this aim involves the use of human subjects thus appropriate approval by RCQ must be in place before the research can be performed. We will resume the research as soon as such approval will be issued (see attached email from Mr. Archie B. Cardwell, and the copy of the extended contract).

Progress towards Aim 2 (Task 2).

Aim 2 of the grant was completed. Using microarray analysis we have determined the candidate growth/tumor suppression pathways involving Hssh3bp1. We also established exon 6 as a candidate region of Hssh3bp1 responsible for growth control *in vitro*. We presented these data in previous year's progress report.

Progress towards Aim 3 (Task 3).

We concentrated our research efforts on Aim 3. The major goal of this aim is to identify potential signal transduction mechanism(s) involving Hssh3bp1. In previous experiments we established the tyrosine phosphorylation site of Hssh3bp1 *in vitro* and determined that Hssh3bp1 expression upregulates c-Abl tyrosine kinase levels in LnCaP cell lines. These studies identified c-Abl tyrosine kinase as a major regulator of Hssh3bp1 function in LnCaP prostate cells. We continued this line of experiments on c-Abl pathway during this reporting period.

Tyrosine 213 is the major phosphorylation site of the recombinant Hssh3bp1 in transfected cells.

To determine whether Hssh3bp1 is phosphorylated *in vivo* we transfected cells with the recombinant Hssh3bp1. Cells were transfected with the wild type and tyrosine mutants of Hssh3bp1; each sequence was tagged with HA epitope at the C-terminus for antibody detection. The Hssh3bp1 mutants included the mutant containing tyrosine 213 to phenylalanine replacement (F213-HA), and the mutant lacking exon 6 sequences (Δ exon6-HA, this mutant lacks exon 6 sequences containing tyrosine 198 and tyrosine 213). Recombinant Hssh3bp1 was immunoprecipitated from transfected cell lysates using the anti-HA antibody and phosphorylation of polypeptides was examined by the anti-phosphotyrosine PY-99 antibody (Fig. 1). The wild type Hssh3bp1 was tyrosine phosphorylated but Δ exon6-HA and F-213 mutants were not. These data suggest that the tyrosine 213 is a major constitutive phosphorylation site of Hssh3bp1 *in vivo*.

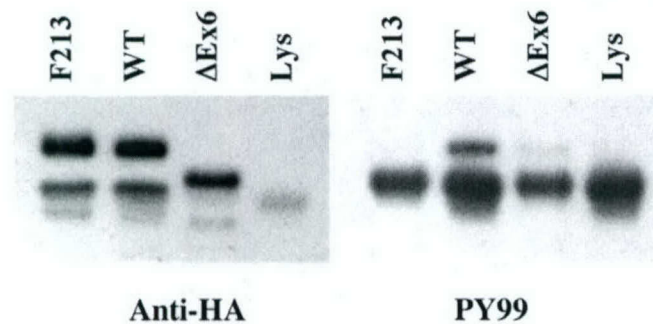
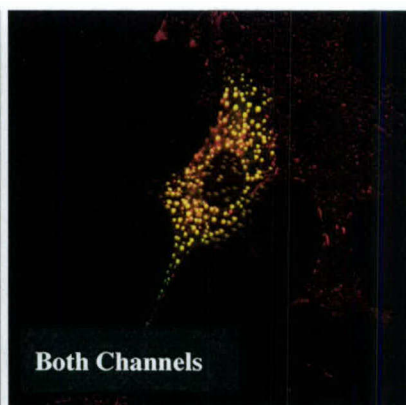
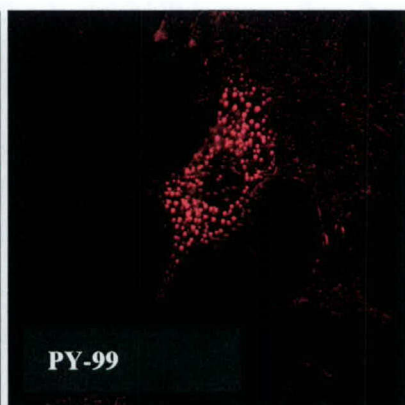
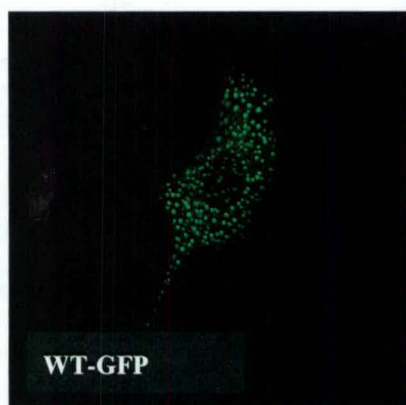


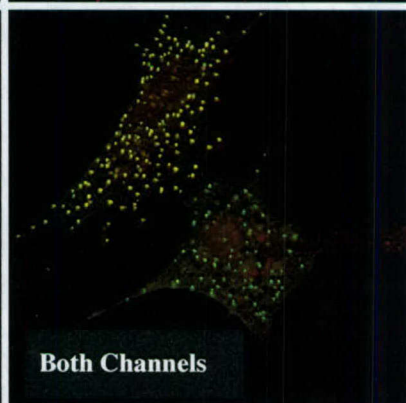
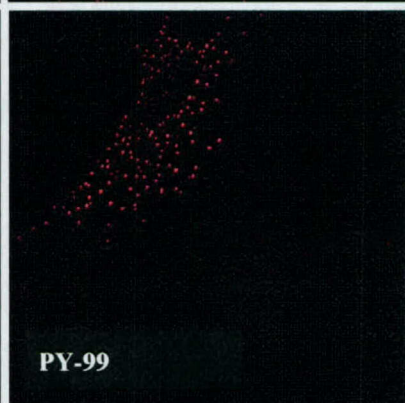
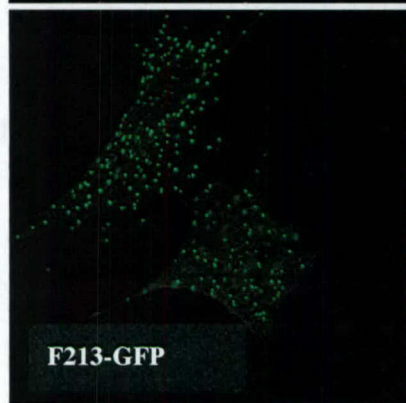
Figure 1. Recombinant *Hssh3bp1* is constitutively phosphorylated on tyrosine 213 in transfected cells. Cells were transfected with recombinant wild type and tyrosine mutants of *Hssh3bp1* tagged at the C-terminus with HA epitope sequence. Following immunoprecipitation with polyclonal anti-HA antibody immunoprecipitated samples were separated on 7% Tris-Tricine polyacrylamide gel and transferred onto nitrocellulose membrane. The membrane was blotted first with the monoclonal antibody to HA (Anti-HA, left panel), following by stripping and blotting with the anti-phosphotyrosine antibody PY-99 (PY-99, right panel). Lane 1, F213-HA; Lane 2, WT-HA; Lane 3, Δ exon6-HA; Lane 4, pEGFP-N2 (vector only control).

Hssh3bp1 accumulates in punctate structures containing phosphorylated tyrosine residues in transfected cells (Fig. 2). Our group demonstrated that upon expression *Hssh3bp1* exhibits very specific localization pattern in transfected NIH 3T3 cells (Xu, J., Ziemnicka, D., Merz, G.S., and L. Kotula. 2000. Human spectrin SH3 domain binding protein 1 regulates macropinocytosis in NIH 3T3 cells. *J Cell Sci.* 113:3805-3814.). Now, we examined whether tyrosine phosphorylation is observed in these structures and whether the phosphorylation is affected by expression of tyrosine mutants of *Hssh3bp1*. We used the same antibody PY-99 as was used for Western blotting for analysis. Phosphorylation was examined in cells transfected with the *Hssh3bp1*-GFP fusion proteins containing the same mutations as in *Hssh3bp1*-HA tagged plasmids evaluated by Western blotting. The transfected cells were co-stained with anti-phosphotyrosine antibody PY-99. We compared phosphorylation of cells transfected with the *Hssh3bp1* mutant containing tyrosine 213 to phenylalanine replacement (F213-GFP), and the *Hssh3bp1* mutant lacking exon 6 sequences (Δ exon6-GFP). The representative data is shown in Figure 2. Preliminary quantitative data indicate that in 50% of cells transfected wild type *Hssh3bp1* (WT-*Hssh3bp1*) the punctate structures were phosphorylated as indicated by red signal in PY-99 plate. In cells transfected with F213-GFP, or in cells transfected with Δ exon6-GFP (not shown) approximately 70% of cells contained non-phosphorylated structures (lack of red signal, middle plate of panel B, lower cell) but some cells (approximately 30%) contained phosphorylated structures (middle panel, upper cell). Interestingly there must be the on/off signal regulating the *Hssh3bp1* phosphorylation because either the particular cell is phosphorylated or not (see Fig.2, panel B). Similar observation i.e. presence or absence of phosphorylation in different transfected cell can be made in cells transfected with the recombinant c-Abl kinase (see panel C). We hypothesize that c-Abl kinase and *Hssh3bp1* phosphorylation may be co-regulated by specific on/off signal. These observations were made in non-synchronized cell culture thus it is possible that the signal for *Hssh3bp1* and c-Abl phosphorylation is dependent on cell cycle.

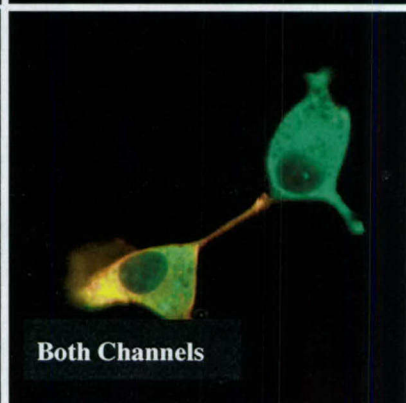
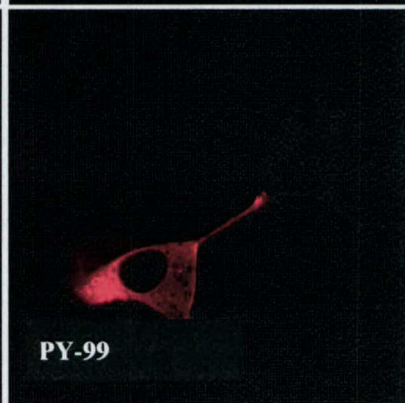
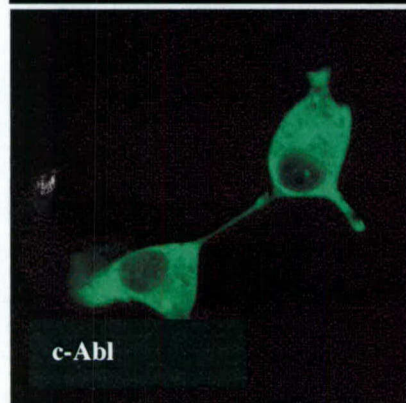
A



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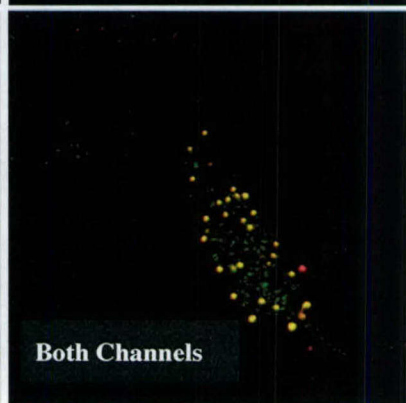
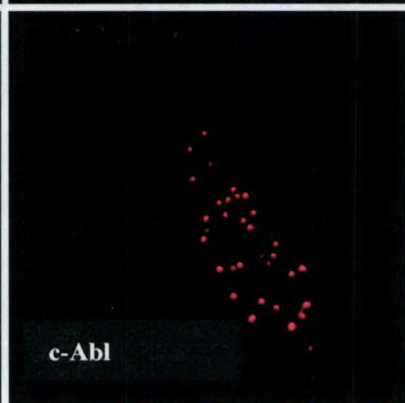
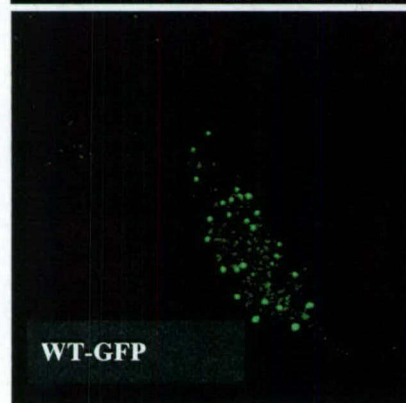


Figure 2. *Phosphorylation of punctate structures containing Hssh3bp1 and colocalization with c-Abl tyrosine kinase in transfected cells.* Cells were transfected with the following plasmids: the recombinant wild type Hssh3bp1 (WT-GFP, panel A); the tyrosine to phenylalanine mutant of residue 213 (F213-GFP, panel B) were expressed as green fluorescent protein (GFP) fusion proteins (panels A and B); the c-Abl tyrosine kinase (c-Abl, panel C); or co-transfected with wild type Hssh3bp1 and the c-Abl tyrosine kinase (panel D). Cells in panels A-C were stained with the anti-phosphotyrosine antibody PY-99 (red channel). Cells in panels C and D were stained with anti-cAbl specific antibody Ab-3 (OP-20, Calbiochem, La Jolla, CA). The red signal in PY-99 plates indicates phosphorylation. *Both channels*, indicates that signal was obtained from green and red lasers simultaneously. Images were obtained by confocal microscopy. The yellow signal in panel D, *Both channels*, indicates protein colocalization.

Recombinant Hssh3bp1 and c-Abl kinase colocalize in transfected cells (Fig. 2).

When plasmids encoding recombinant Hssh3bp1 and c-Abl kinase are co-transfected into cultured cells both proteins colocalize in punctate structures in cells (see Figure 2, panel D). Interestingly, when expressed alone c-Abl kinase show diffuse cytoplasmic staining. These data indicate that Hssh3bp1 recruits c-Abl into the punctate structures. These structures are likely to be macropinocytic vesicles (Xu et al., 2000). These data suggest that Hssh3bp1 play a role in localization of c-Abl in cells. We will explore feasibility of experiments to determine whether c-Abl is active or inactive in the punctate structures. These experiments are likely to be part of another grant application to DOD.

Key Research Accomplishments

- ◆ Tyrosine 213 is the major phosphorylation site of recombinant Hssh3bp1 in transfected cells.
- ◆ Hssh3bp1 affects phosphorylation of punctate structures in transfected cells.
- ◆ Hssh3bp1 may play a role in localization of c-Abl in cells.

Reportable Outcomes

Development of stable prostate cell lines expressing Hssh3bp1.

The LnCap and PC3 cell lines expressing Hssh3bp1 established in our laboratory will be available to scientific community upon publication of the results of this work

Invited talk: Invited talk at the international meeting "Emerging Pathways in Cytoskeletal Communication", June 5-9, 2004, Umeå, Sweden. The talk was entitled: "Phosphorylation of Abi-1/Hssh3bp1 by c-Abl tyrosine kinase"

Manuscript describing the data presented in current and a previous report is under preparation.

Conclusions

Three major conclusion of the presented progress of work are:

1. Tyrosine 213 is the major phosphorylation site of recombinant Hssh3bp1 in transfected cells.
2. Hssh3bp1 affects phosphorylation of punctate structures in transfected cells.
3. Hssh3bp1 may play a role in localization of c-Abl in cells.

This year we have further characterized phosphorylation of Hssh3bp1. Tyrosine 213 is phosphorylated on recombinant Hssh3bp1 transfected into cells. Thus it is very likely that tyrosine 213 is the phosphorylation site on endogenous Hssh3bp1. Tyrosine phosphorylation of c-Abl and Hssh3bp1 is likely to co-regulated in cells. Colocalization of Hssh3bp1 with Abl kinase in cells suggests that Hssh3bp1 may control localization and function of Abl *in vivo*. In fact these types of experiments provide possibility of looking regulation of Abl activity in these structures both *in vitro* and in live cells (these experiments will be pursued but are beyond the scope of this grant research). Identification of the major phosphorylation site of Hssh3bp1 by c-Abl tyrosine kinase provides both starting point for protein biochemical studies of Hssh3bp1 in prostate tumors as well as a specific change (or marker) that can be compared in tumor vs. normal tissue.

References

1. Xu, J., Ziemnicka, D., Merz, G.S., and L. Kotula. 2000. Human spectrin SH3 domain binding protein 1 regulates macropinocytosis in NIH 3T3 cells. *J Cell Sci.* 113:3805-3814.

Appendix

Copies of the Assistance Agreement and Email from Mr Archie Cardwell Jr. , the grant specialist.

ASSISTANCE AGREEMENT

AWARD TYPE: <input checked="" type="checkbox"/> GRANT (31 USC 6304) <input type="checkbox"/> COOPERATIVE AGREEMENT (31 USC 6305) <input type="checkbox"/> OTHER TRANSACTION (10 USC 2371)			
AWARD NO: DAMD17-01-1-0096 Modification P00001		EFFECTIVE DATE See Grants Officer Signature Date Below	
		AWARD AMOUNT \$391,663.00	
Page 1 of 2 Archie Cardwell Jr. (301) 619-2630 Fax: (301) 619-3002			
PROJECT TITLE: The Role of Human Spectrin SH3 Domain Binding Protein 1 (HSSH3BP1) in Prostatic Adenocarcinoma.			
CFDA 12.420			
PERFORMANCE PERIOD: 20 August 2001- 19 September 2005 (Research ends 19 August 2005)		PRINCIPAL INVESTIGATOR: Leszek Kotula, M.D.	
AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-W 820 Chandler St. Fort Detrick Maryland 21702-5014		PAYMENTS WILL BE MADE BY: EFT:T Army Vendor Pay DFAS-SA-FPA (888) 478-5636 500 McCullough Avenue San Antonio, TX 78215-2100	
DUNS No: 07-991-9916		TIN No:	
AWARDED TO: Research Foundation for Mental Hygiene, Inc 44 Holland Avenue Albany, NY 12229		(SEE PARAGRAPH TITLED "PAYMENTS" FOR INSTRUCTIONS) REMIT PAYMENT TO: N/A	
ACCOUNTING AND APPROPRIATION DATA: NO CHANGE			
SCOPE OF WORK: A. The period of performance of this Assistance Agreement is hereby extended for one year at no additional cost to the Government. The new period of performance is 20 August 2001 through 19 September 2005 with research to end on 19 August 2005. B. See Page 2 of 2 for prohibitions			
RECIPIENT		GRANTS OFFICER	
ACCEPTED BY:		UNITED STATES OF AMERICA	
SIGNATURE		SIGNATURE	
NAME AND TITLE		NAME AND TITLE	
DATE		DATE	
		Joseph S. Little GRANTS OFFICER	
		12 Aug 04	

Assistant Agreement DAMD17-01-1-0096

Modification P00001

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C. PROHIBITION OF USE OF HUMAN SUBJECTS (NOV 2000) (USAMRAA)

Notwithstanding any other provisions contained in this award or incorporated by reference herein, the recipient is expressly forbidden to use or subcontract for the use of human subjects in any manner whatsoever. In the performance of this award, the recipient agrees not to come into contact with, use or employ, or subcontract for the use or employ of any human subjects for research, experimentation, tests or other treatment under the scope of work as set out in the award

D. PROHIBITION OF USE OF HUMAN ANATOMICAL SUBSTANCES (NOV 2000) (USAMRAA)

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Notwithstanding any other provisions contained in this award or incorporated by reference herein, the recipient is expressly forbidden to use or subcontract for the use of laboratory animals in any manner whatsoever without the express written approval of the Grants Officer.

Hello Dr. Kotula,

Thank you for the accolades.

To answer your question, all the prohibitions will remain in place until RCQ advises me they have reviewed everything and it is safe to them.

Sincerely,

Archie B. Cardwell Jr.
Grant/Contract Specialist
USAMRAA
BLDG 820, Chandler Street
Fort Detrick, MD 21702
301-619-2630
FAX: 301-619-3002
archie.cardwell@amedd.army.mil

From: Kotula, Leszek [mailto:LKotula@NYBloodCenter.org]
Sent: Tuesday, September 14, 2004 1:59 PM
To: Cardwell, Archie B Mr USAMRAA
Cc: CORCCMC@omh.state.ny.us
Subject: ASSISTANCE AGREEMENT # DAMD17-01-1-0096, LESZEK KOTULA
Importance: High

Dear Mr Archie Cardwell,

I would like to thank you for extending the grant for one year. You did a great job in doing it so swiftly. Thanks again.

I would like to ask you for clarification of the prohibitions imposed on the grant (human subjects, use of human anatomical substances, animals). I understand that these prohibitions are in effect through the end of the grant. Am I correct? Please let me know.

Best regards,

Leszek Kotula
Lab of Cell Signaling
NYBC
310 E. 67th Street
New York, NY 10021

New York Blood Center
For more information or to make an appointment we invite you to call 1-800-933-BLOOD
or to visit our website www.nybloodcenter.org

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